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The Development of Nitroxide Based Coatings for Biofilm Remediation

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14. ABSTRACT
In this research, the PI initially aimed to investigate the chemistry of nitroxides within biofilms in order to understand the mechanism by which nitroxides inhibit and disperse biofilms. The PIs then planned to develop new anti-biofilm agents based on nitroxides, explore the application of new chemistries for the immobilisation of nitroxides to relevant substrates and evaluate the effects of these functionalised scaffolds on biofilm growth. The PI have synthesized a water soluble profluorescent nitroxide for biofilm imaging. This nitroxide was shown to eradicate an existing biofilm in conjunction with antibiotic use, with excellent results, indicating that these compounds are good targets for future research. Additional research shows that the surface binding nitroxides are most likely inactive, suggesting that the anti-biofilm actions is intracellular.

The research resulted in 1 peer reviewed publication, 3 conference presentations, and 1 manuscript in progress. They also have an excellent collaboration with AFRL/RX (Wendy Goodson) and will continue to work on testing compounds provided by this research.

15. SUBJECT TERMS
Biofilms, Nitroxides, Biofuels, Organic Synthesis, Coatings

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Annual Report for AOARD Grant 15IOA087-154020 "The Development of Nitroxide Based Coatings for Biofilm Remediation"

7th June 2017

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Abstract: Bacterial biofilms are a major problem in a number of environmental, industrial and medical applications. They cause significant risks to human health and present an enormous economic burden to society. An emerging strategy to combat biofilm formation and growth is to use small molecules that act through non-microbicidal mechanisms to inhibit and/or disperse biofilms. Nitroxides have shown potential in this regard, demonstrating both biofilm inhibition and dispersal properties. This project aimed to develop novel nitroxide-based anti-biofilm agents and incorporate these compounds into surface coatings. Our results have shown that the combination treatment of mature *P. aeruginosa* and *E. coli* biofilms with nitroxides and ciprofloxacin (an antibiotic) led to almost complete eradication of bacteria. Hybrid molecules comprised of different hindered nitroxides linked to the piperazinyl secondary amine of ciprofloxacin via amide or tertiary amine linkers were synthesized and displayed significant biofilm dispersal and eradication activities. The activity of surface-tethered nitroxides was also investigated. Silicon wafers were functionalized with nitroxides using alkoxysilane chemistry. Evaluation of the potential biofilm inhibitory action of the surfaces in a static assay with *P. aeruginosa* showed little effect, potentially indicating that the previously observed anti-biofilm activity in solution is intracellular and requires release of the nitroxide from the surface.

Introduction: The global aim of this project was to develop smart polymeric coatings for materials to discourage bacterial attachment and ensure long term control over biofilm growth and proliferation. This fundamental work will find application in a number of areas including combating the presence of biofilms in fuel storage tanks. Hydrocarbon utilising micro-organisms (HUM Bugs) degrade fuels, block filters and injectors and corrode fuel storage tanks and pipes. Biocides are currently used to remove bacterial growth present in aircraft fuel systems however this approach is ineffective if the bacteria reside in a biofilm. In this case, tanks must be drained and mechanically cleaned. This process is time consuming, labor intensive and costly. The surface coatings developed as part of this research would allow contamination to be controlled before the hydrocarbon utilising bacteria could cause significant problems. The proposed smart polymeric coatings will use a novel mode of action to facilitate improved control over biofilm growth and should therefore have a significant impact in this area of application.

In this work, we initially aimed to investigate the chemistry of nitroxides within biofilms in order to understand the mechanism by which nitroxides inhibit and disperse biofilms. We then planned to develop new anti-biofilm agents based on nitroxides, explore the application of new chemistries for the immobilisation of nitroxides to relevant substrates and evaluate the effects of these functionalised scaffolds on biofilm growth. The five fundamental challenges initially outlined in this work were:

1. Over what period of time do nitroxides continue to inhibit or disperse bacterial biofilms? Is the nitroxide oxidized or reduced during this process and is this chemistry reversible (i.e. could the nitroxide have an infinite activity because of redox cycling?)

2. Does the use of a nitroxide in combination with an antibiotic kill bacteria residing in a biofilm? Are the individual activities of the nitroxide and antibiotic retained when they are combined within the

one molecule?

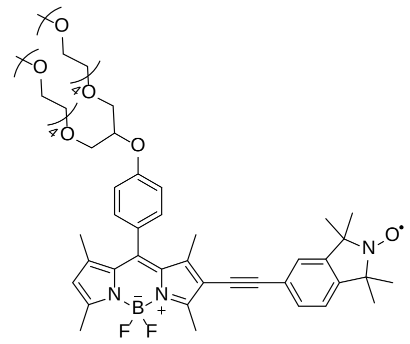
3. Is the anti-biofilm activity of nitroxides in solution conserved when they are immobilized to a surface? Is the length of tethering linker important?

4. If surface bound nitroxides are shown to have lower activity, how can the nitroxide-antibiotic hybrid molecules be released from a surface with initial high concentrations followed by release in response to a growing biofilm?

5. How can nitroxides be immobilized onto a range of materials (such as titanium, stainless steel, aluminium etc.)?

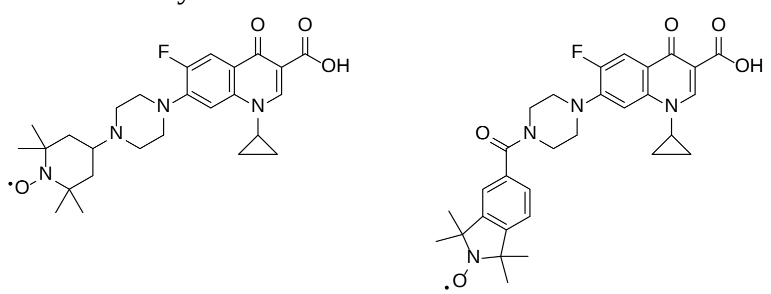
Experiment: Our approaches used to address each of the fundamental challenges are outlined below:

Challenge 1: Synthesize a water soluble profluorescent nitroxide which will enable the redox states of the nitroxide within the biofilm to be studied by EPR and imaged by spectrofluorimetry.



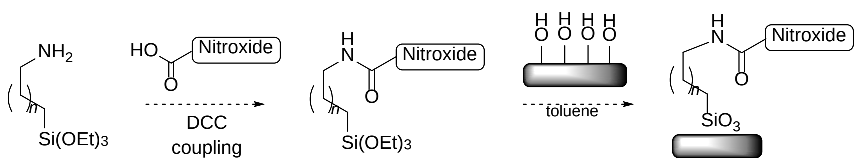
Water soluble BODIPY-based profluorescent nitroxide target

Challenge 2: Co-PI Hancock's group to investigate if a nitroxide in combination with an antibiotic will kill biofilm bacteria. Ciprofloxacin-antibiotic hybrids need to be synthesized, fully characterized and then evaluated for activity in biofilm assays.



Amine- and amide-linked ciprofloxacin-nitroxide hybrid targets

Challenge 3: Silicon wafers will be functionalized with nitroxides using trialkoxysilane reagents. Trialkoxysilane-containing nitroxides will be prepared.



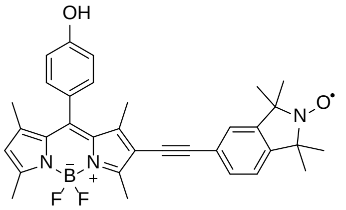
Proposed synthetic route to nitroxide-bearing alkoxysilane and silicon wafer functionalization

Challenges 4 and 5: Nitroxide-antibiotics to be attached through cleavable linkers to surfaces.

Nitroxide-antibiotics tethered through cleavable linkers will be incorporated into poly(ethylene)glycol based random copolymers.

Results and Discussion:

Challenge 1: Efforts to synthesize a water soluble profluorescent nitroxide for biofilm imaging of redox status are ongoing. The precursor **1** has been prepared and is now ready for the attachment of the water solubilising tetra(ethylene)glycol swallow tail. Once this probe is in hand, its fluorescent properties will be examined and biofilm imaging experiments can be performed to determine the redox chemistry of the nitroxide moiety within a biofilm.



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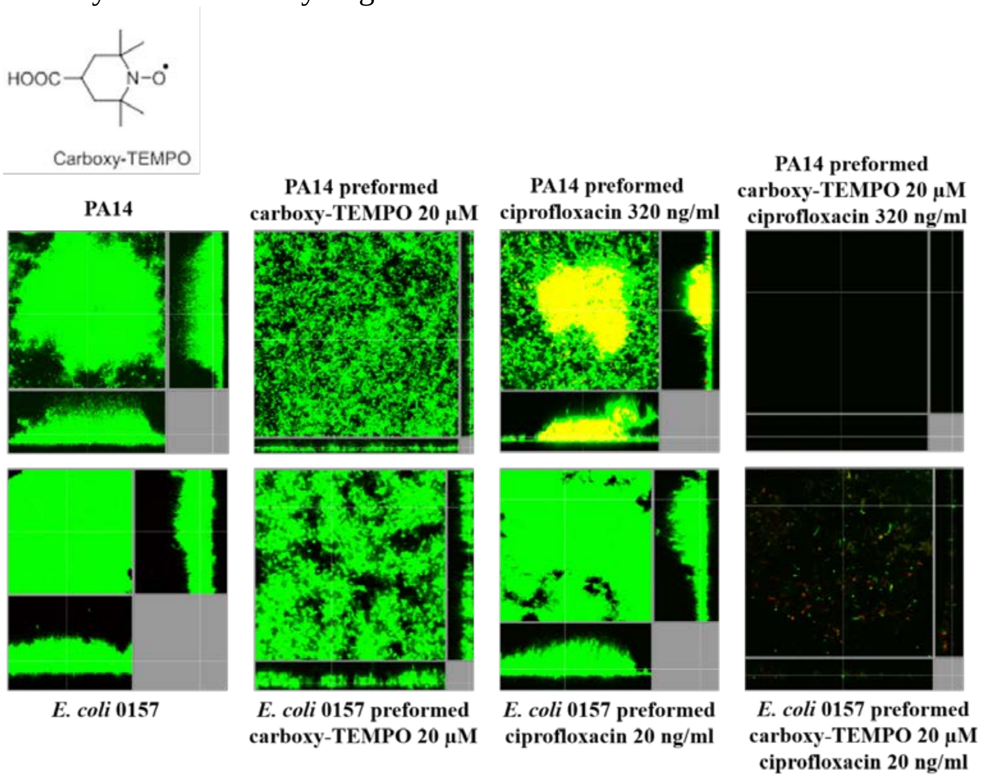
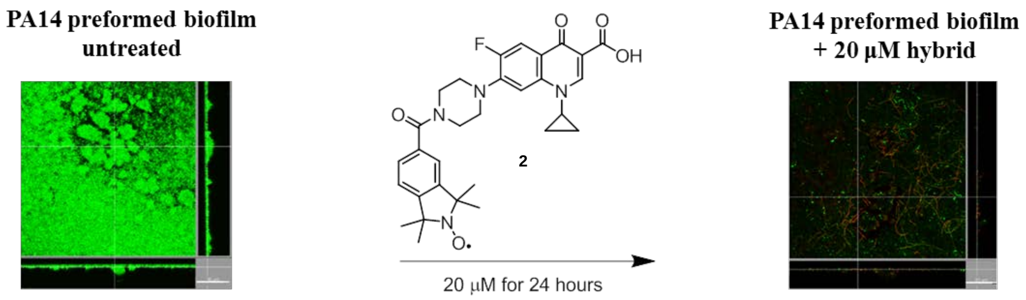
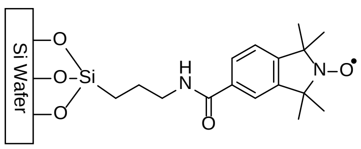
Challenge 2: Co-PI Hancock's group has found that the use of a nitroxide in combination with an antibiotic (ciprofloxacin) can eradicate an existing biofilm. This result is extremely significant as it provides a means to circumvent the extreme resistance of biofilms to antibiotics and suggests that the nitroxide-antibiotic hybrids are worthy targets.

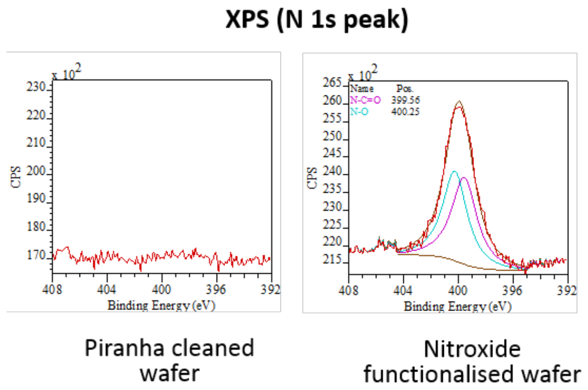


In this regard, we have synthesized a range of nitroxide-ciprofloxacin conjugate molecules, using several nitroxide classes and different linkages between the two moieties. Compound **2** was shown to be the most promising candidate in biological evaluations conducted by Co-PI Hancock's group, eradicating 99% of the biofilm after treatment at 20 µM for 24 hours in a flow cell. These compounds represent the first dual-action nitroxide-antimicrobial agents, which harness the dispersal properties of the nitroxide moiety to circumvent the well-known resistance of biofilms to treatment with antimicrobial agents.



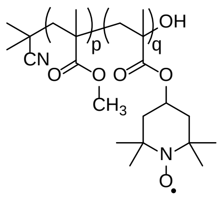
Challenge 3: In order to determine if the known activity of nitroxides against biofilms is retained on a surface, nitroxide functionalized surfaces were targeted. As a model system, we prepared Si wafers functionalized with nitroxides using alkoxysilane chemistry. The surfaces have been characterized by XPS (X-ray photoelectron spectroscopy), contact angle and ToF-SIMS (time of flight secondary ion mass spectrometry). In the XPS, the presence of nitrogen on the surface was observed in the nitroxide-functionalized surfaces (deconvolution revealed both N-C=O and N-O peaks). The contact angle increased from 33^{o} to 78^{o} following nitroxide functionalization, consistent with a more hydrophobic surface. The ToF-SIMS, an NO- fragment is observed at 30 *m/z*, showing the presence of the nitroxide moiety.





Preliminary analysis of these surfaces for inhibition of biofilm growth in a static assay has shown that the surfaces have little effect on biofilm formation. This result is very important as it informs future scaffold development by revealing that the surface-bound nitroxides are most probably inactive, which suggests that the anti-biofilm action of nitroxides is intracellular.

Challenges 4 and 5: Copolymer blends using PMMA and the TEMPO-containing methacrylate monomer TEMPOMA (in different ratios) have been prepared and characterized. The polymers have been spin coated onto silicon wafer surfaces and sent to AFRL for biofilm inhibition testing. Once results are obtained, we should have a better understanding of how the ratio of nitroxide incorporated effects the anti-biofilm activity. We then plan to incorporate PEG chains into the copolymer to help limit bacteria adhesion and use cleavable linkages for nitroxide release.



List of Publications and Significant Collaborations that resulted from your AOARD supported project: In standard format showing authors, title, journal, issue, pages, and date, for each category list the following:

a) papers published in peer-reviewed journals,

1) Verderosa, A. D.; Mansour, S. C.; de la Fuente-Núñez, C.; Hancock, R. E. W.; Fairfull-Smith, K. E., "Synthesis and Evaluation of Ciprofloxacin-Nitroxide Conjugates as Anti-Biofilm Agents", *Molecules* **2016**, *21*.

b) papers published in non-peer-reviewed journals or in conference proceedings,
N/A

c) conference presentations,

1) Kathryn E. Fairfull-Smith, oral presentation, "Applications of Nitroxides in Materials Science", Air Force Research Laboratory, Dayton, Ohio, USA, February 2016.
2) Kathryn E. Fairfull-Smith, invited Early Career Researcher speaker, "Nitroxide-Containing Materials for Biofilm Remediation", 36th Australasian Polymer Symposium, Lorne, Victoria, Australia, November 2016.
3) Kathryn E. Fairfull-Smith, oral presentation, "Nitroxide-Containing Agents for Biofilm Remediation", 24th Annual Meeting - Society for Free Radical Research Australasia, Gold Coast, Australia, December 2016.

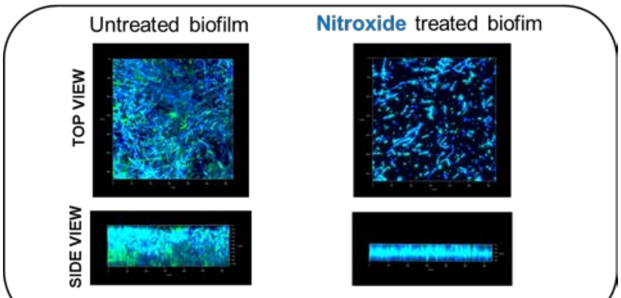
d) manuscripts submitted but not yet published

1) Verderosa, A. D.; de la Fuente-Núñez, C.; Mansour, S. C.; Cao, J.; Lu, T. K.; Hancock, R. E. W.; Fairfull-Smith, K. E., "Ciprofloxacin-Nitroxide Hybrids with Potential for Biofilm Control", *European Journal of Medicinal Chemistry*, **2017**, in revision.

e) provide a list any interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work.

We are building a collaboration with Dr Caitlin Bowjanowski and Wendy Goodson (AFRL). PI Fairfull-Smith visited the AFRL (Dayton, Ohio) in February 2016 on a Windows on Science grant.

- Dr Caitlin Bowjanowski has performed initial work which demonstrates that nitroxides have potential to disperse yeast and fungal biofilms.



Yarrowia lipolytica, cell walls stained blue

- Surfaces spin coated with copolymer blends prepared using PMMA and the TEMPO-containing methacrylate monomer TEMPOMA are currently being assessed for biofilm inhibitory action at AFRL.

- Nitroxides and antifungal agents will be tested as a potential combination therapy to overcome fungal biofilm resistance to antifungals/biocides.

- Please note: The current assessment of small molecules and functionalized surfaces provided to AFRL for anti-fungal evaluation in 2016 has stalled as AFRL scientist Dr Caitlin Bowjanowski has been devoting her time to completing her PhD. She plans to resume testing in September 2017.